

# Novel and Recurrent Mutations in the Genes Encoding Keratins K6a, K16 and K17 in 13 Cases of Pachyonychia Congenita

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Thirteen patients with pachyonychia congenita types 1 and 2 were studied, two of which had a family history of pachyonychia and 11 of which were sporadic cases. Heterozygous mis-sense or small in-frame insertion/deletion mutations were detected in the genes encoding keratins K6a, K16, and K17 in all cases. Three novel mutations, F174V, E472K, and L469R were found in the K6a gene. Two novel mutations, M121T and L128Q were detected in K16. Similarly, three novel mutations, L95P, S97del, and L99P were found in K17. In addition, we identified recurrent mutations N171del (three instances) and F174S in K6a and R94H in K17. Analysis of both phenotype and genotype data led to the following conclusions: (i) K6a or K16 mutations produce the pachyonychia congenita type 1 phenotype, whereas

K17 (or K6b) mutations cause pachyonychia congenita type 2; (ii) the presence of pilosebaceous cysts following puberty is the best indicator of pachyonychia congenita type 2; (iii) prepubescent patients are more difficult to classify due to the lack of cysts; and (iv) natal teeth are indicative of pachyonychia congenita type 2, although their absence does not preclude the pachyonychia congenita type 2 phenotype. This study establishes useful diagnostic criteria for pachyonychia congenita types 1 and 2, which will help limit unnecessary DNA analysis in the diagnosis and management of this genetically heterogeneous group of genodermatoses. **Key words:** genodermatoses/keratin/mutation/nail dystrophy/pachyonychia congenita. *J Invest Dermatol* 117:1391–1396, 2001

**K**eratin intermediate filaments are the main stress-bearing cytoskeletal system within the cytoplasm of epithelial cells (Corden and McLean, 2001). Keratins form heteropolymers consisting of specific pairs of type I and type II proteins that are expressed in a tissue-specific and differentiation-specific fashion (Lane, 1993). Their assembly into 10 nm intermediate filaments is mediated by their central coiled-coil  $\alpha$ -helical rod domains (Quinlan *et al*, 1994). Conserved sequences at both ends of the rod domain, termed the helix boundary motifs, are known to be involved in end-to-end interactions in filament assembly (Steinert *et al*, 1993; Herrmann *et al*, 2000). In recent years, genetic mutations affecting the assembly and/or integrity of keratin intermediate filaments have been shown to be the cause of a wide variety of human genetic disorders of keratinization (Fuchs and Cleveland, 1998; Irvine and McLean, 1999). The majority of keratin mutations are dominant acting and mostly involve mis-sense or small in-frame insertion/

deletion mutations clustering at the ends of the rod domain of the keratin molecule (Corden and McLean, 1996).

Pachyonychia congenita (PC) is an autosomal dominant group of ectodermal dysplasias characterized by hypertrophic nail dystrophy, hyponychial keratosis, palmoplantar keratoderma, and other ectodermal changes (Jadassohn and Lewandowsky, 1906; Jackson and Lawler, 1951). Two clinical subtypes of PC have been described: the Jadassohn–Lewandowsky or PC-1 type (OMIM no. 167200) and the Jackson–Lawler or PC-2 variant (OMIM no. 167210). In PC-1, oral leukokeratosis, palmoplantar keratoderma, and follicular keratosis may be observed (Jadassohn and Lewandowsky, 1906). The PC-2 subtype is characterized by the presence of additional features, including multiple pilosebaceous cysts, neonatal teeth, and pili torti (Jackson and Lawler, 1951). In PC-2, oral leukokeratosis can occur (Smith *et al*, 1998) but appears to be less frequently observed than in PC-1. In our experience, the presence of widespread pilosebaceous cysts following puberty is the best distinguishing feature of PC-2. Histologically, these cysts are heterogeneous: some the result of infundibular hyperkeratosis and others are steatocysts (Munro *et al*, 1994).

PC-1 has been shown to be caused by mutations in the genes encoding keratins K16 (McLean *et al*, 1995) and K6a (Bowden *et al*, 1995). These keratins are coexpressed in the epithelial tissues

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affected in PC-1 (Weiss *et al*, 1984). In contrast, PC-2 has been shown to result from mutations in the K17 gene (McLean *et al*, 1995), a keratin expressed in the tissues affected in PC-2 (Trojanovsky *et al*, 1989; McGowan and Coulombe, 1998). Recently, a mutation in the K6b gene was reported in an extensive family presenting with the PC-2 phenotype (Smith *et al*, 1998). This mutation, together with coexpression data demonstrated that K6b is the expression partner of K17 in the tissues affected in PC-2 (Smith *et al*, 1998).

Here, we have analyzed 13 kindreds with PC and have found eight novel and five recurrent keratin mutations.

## MATERIALS AND METHODS

**Sample collection** Genomic DNA samples were extracted from peripheral blood lymphocytes of the patients by standard methods.

**Mutation detection and confirmation** PCR primers and programs used for specific amplification of the mutation hotspot regions of the genes encoding K6a and K16 without coamplification of homologous pseudogenes and/or isogenes, were described previously (Smith *et al*, 1999a, b). Exon 1 of the K17 gene was specifically amplified as reported previously (Covello *et al*, 1998). The full-length functional K17 gene (*KRT17A*) was specifically amplified using primers K17P8 and K17P13 (**Table I**) using standard PCR buffer containing 1.5 mM MgCl<sub>2</sub> (Perkin-Elmer, Foster City, CA) and 4% dimethyl sulfoxide. This reaction was subjected to a "hot start" with 1 U of Long Range PCR enzyme mix (Boehringer, Mannheim, Germany) and the following PCR conditions were used: (94°C 5 min) × 1; (94°C 30 s, 58.5°C 1 min, 72°C 2.5 min) × 34; (72°C 5 min) × 1. Mutation detection was performed by direct sequencing of PCR products using the amplification oligonucleotides or additional internal primers. Sequences were compared with that of an unaffected, unrelated control sample. Sequence analysis was performed using Big Dye terminator chemistry on an ABI 377 genetic analyzer (Perkin-Elmer).

For the novel mutations, we performed a series of restriction enzyme digestions of short PCR fragments carrying the mutations and of 50 normal unrelated controls to exclude these sequence changes as

polymorphisms in the normal population. In the case of K16, a short PCR fragment specific for exon 1 of the functional *KRT16A* gene was used. In the case of K6a and K17 genes, nested PCR was used to circumvent pseudogene and/or isogene interference with specific PCR fragments generated as above. Short fragments appropriate for digestion were produced by reamplification of a 1:1000 dilution of the full-length PCR products. For confirmation of K6a mutation F174V and K17 mutation S97del, mismatch primers were used to create new restriction sites dependent on the presence of the mutation. In all other cases, naturally occurring sites were exploited. The PCR reactions and enzymes used in this analysis, together with the fragment sizes generated from digestion of normal and mutant alleles are outlined in **Table II**. The PCR primers used in this analysis and their optimized annealing temperatures are listed in **Table II**. Primer sequences are listed in **Table I**.

## RESULTS

**Case reports** In this study we have collected 13 patients, clinically heterogeneous, diagnosed as PC-1 (**Table III**, patients 1–9) and PC-2 (**Table III**, patients 10–13). In two cases (patients 6 and 12), there was a clear family history of pachyonychia with typical features of autosomal dominant inheritance. All other patients were sporadic cases of pachyonychia. Using the presence or absence of pilosebaceous cysts as the key diagnostic feature, we initially classified the 13 patients into the PC-1 and PC-2 categories, as shown in **Table III**. It should be noted that these cysts only appear at puberty and so younger patients are more difficult to classify. As can be seen from **Table III**, other features often used to distinguish the two groups, such as oral leukokeratosis in PC-1 or natal teeth in PC-2, are not fully expressed and therefore are less reliable as diagnostic indicators. The presence of natal teeth, however, is a useful indicator of PC-2 in prepubescent patients, although the absence of natal teeth does not preclude a diagnosis of PC-2. It should be noted that in some cases of PC-1, follicular hyperkeratosis can occur, such as in patient 7 here (**Table III**). These lesions may be mistaken for pilosebaceous cysts and therefore lead to misdiagnosis as PC-2.

The severity of palmoplantar keratoderma varied widely within both PC types. Most were mildly affected with keratoderma confined only to the pressure areas of plantar epidermis with little or no palmar involvement. Some cases, however, notably patient 3, had severe diffuse keratoderma of both palms and soles, resembling epidermolytic palmoplantar keratoderma. One case (patient 6), suffered hyperkeratotic lesions of the conjunctiva, when this tissue was stressed by use of contact lenses. This feature has not previously been reported in pachyonychia.

**Novel and recurrent mutations in PC** Patients were screened for mutations in the K6a, K16, and K17 genes, which have been previously associated with the PC-1 and PC-2 types of the disease (Bowden *et al*, 1995; McLean *et al*, 1995; Smith *et al*, 1998). In the patients diagnosed as PC-1, the entire coding regions of the K6a and K16 genes were amplified by the long-range PCR protocols described previously (Smith *et al*, 1999a, b). K6a mutations were detected in seven of the PC-1 individuals examined (patients 1–7;

**Table I. Primers used for mutation detection/confirmation**

Primer name	Sequence 5'-3'
K6aHincII <sup>a</sup>	ACTGGCTCACCTTGTTCGATGAAGGAGTCAA
K6ap4	AGGCATCCAAGAGGTCACCG
K6ap8	AGATGGCCCTCAAGGATGCC
K6ap9	GGGAGGGTGAGACCTCAGAGAGCT
K16sp2	AGGGCTCCTGCGGCATCGGA
K16sp6	TACACCAAAGTACCCACCTTGTT
K17SfiI <sup>a</sup>	GTTGGCCTCTCCAGGCACGACCTTGGCC
K17P4	GGTACCAGTCACGGATCTTCA
K17P8	GCCTATAAAGGAAGCGGGC
K17P13	ACAAGCTTTATTGTCATCAGGCAA

<sup>a</sup>Mismatch bases used to create new enzyme sites are underlined.

**Table II. PCR reactions used for restriction enzyme analysis**

Mutation (protein; DNA)	Primers, F & R	Annealing	PCR type	PCR size	Enzyme	Normal bands	Mutant bands
K6a F174V; 520T > G	K6ap4, K6aHincII	59°C	Nested	152 bp	HincII <sup>a</sup> (+)	130, 22 bp	102, 28, 22 bp
K6a L469R; 1406T > G	K6ap8, K6ap9	58°C	Nested	379 bp	BpmI (–)	205, 117, 57 bp	322, 57 bp
K6a E472K; 1414G > A	K6ap8, K6ap9	58°C	Nested	379 bp	BseRI (–)	202, 177 bp	379 bp
K16 M121T; 362T > C	K16sp2, K16sp6	57.5°C	Direct	506 bp	NlaIII (–)	320, 186 bp	506 bp
K16 L128Q; 383T > A	K16sp2, K16sp6	57.5°C	Direct	506 bp	StuI (+)	506 bp	342, 164 bp
K17 L95P; 284T > C	K17P8, K17P4	58°C	Nested	437 bp	NaiI (+)	437, 25 bp	359, 78, 25 bp
K17 S97del; 289delCCT	K17P8, K17SfiI	58.5°C	Nested	427 bp	SfiI <sup>a</sup> (+)	428 bp	393, 32 bp
K17 L99P; 296T > C	K17P8, K17P4	57°C	Nested	437 bp	NaiI (+)	437, 25 bp	371, 66, 25 bp

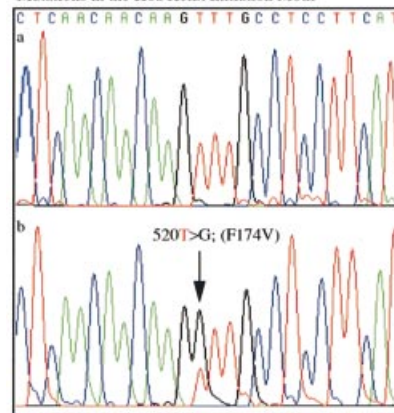
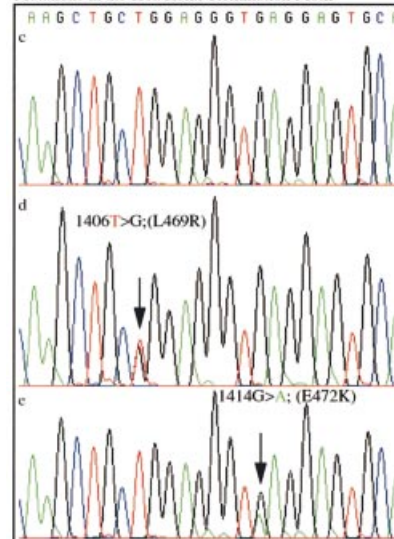
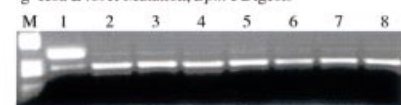
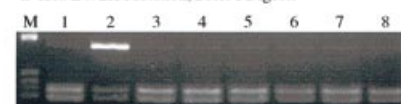
<sup>a</sup>New restriction site created by primer mismatch.

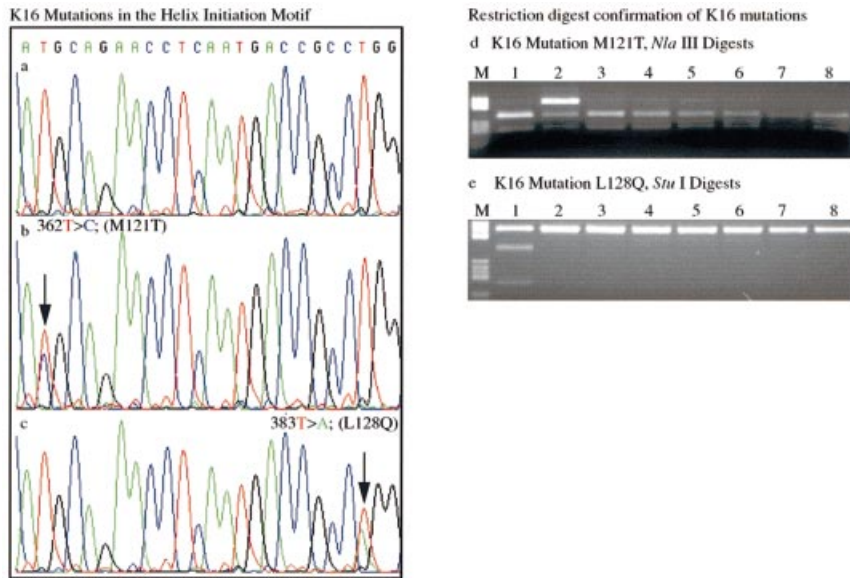
**Table III. Phenotype and genotype data for pachyonychia patients studied**

Patient	PC type	Inheritance	PC	PPK	Oral <sup>a</sup>	Ocular <sup>b</sup>	FH <sup>c</sup>	Cysts <sup>d</sup>	NT <sup>e</sup>	Mutation <sup>f</sup>
1	PC-1	Sporadic	+	–	–	–	–	–	–	<b>K6a F174V</b> (1A domain F10V)
2	PC-1	Sporadic	+	+	+	–	–	–	–	<b>K6a E472K</b> (2B domain E117K)
3	PC-1	Sporadic	+	+	+	–	–	–	–	<b>K6a L469R</b> (2B domain L114R)
4	PC-1	Sporadic	+	–	–	–	–	–	–	K6a N171del (1A domain N8del)
5	PC-1	Sporadic	+	–	+	–	–	–	–	K6a F174S(1A domain F10S)
6	PC-1	Familial	+	+	+	+	–	–	–	K6a N171del (1A domain N8del)
7	PC-1	Sporadic	+	+	+	–	+	–	–	K6a N171del (1A domain N8del)
8	PC-1	Sporadic	+	+	+	–	–	–	–	<b>K16M121T</b> (1A domain M4T)
9	PC-1	Sporadic	+	+	+	–	–	–	–	<b>K16L128Q</b> (1A domain L11Q)
10	PC-2	Sporadic	+	+	–	–	–	+	–	<b>K17L95P</b> (1A domain L11P)
11	PC-2	Sporadic	+	+	–	–	–	+	–	<b>K17S97del</b> (1A domain S13del)
12	PC-2	Familial	+	+	–	–	–	+	+	<b>K17L99P</b> (1A domain L15P)
13	PC-2	Sporadic	+	+	–	–	–	+	–	K17: R94H (1A domain R10H)

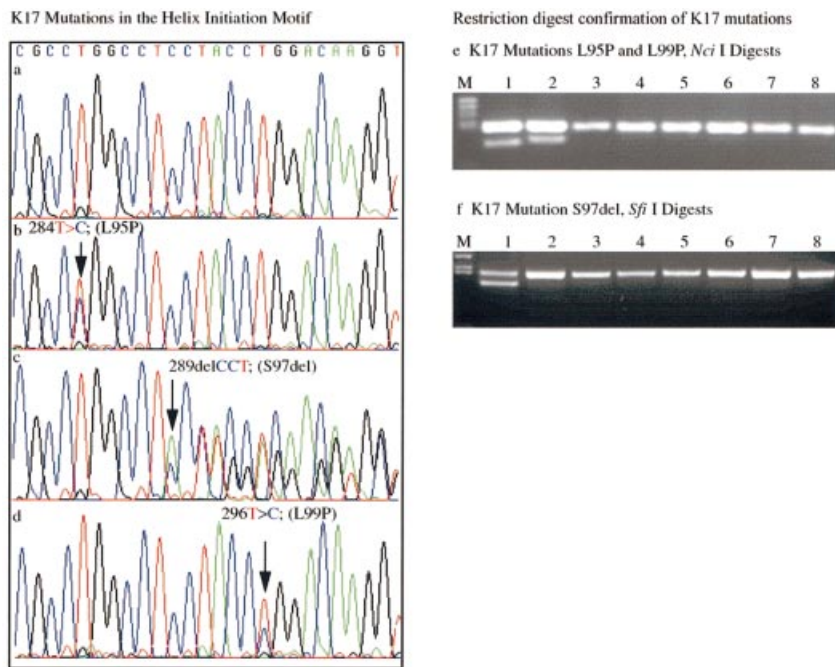
<sup>a</sup>Oral leukokeratosis.<sup>b</sup>Keratosis of the ocular mucosa.<sup>c</sup>Follicular hyperkeratosis.<sup>d</sup>Piloosebaceous cysts i.e. steatocysts and/or eruptive vellus hair cysts.<sup>e</sup>Natal teeth.<sup>f</sup>Numbers in parenthesis refer to the 1A or 2B domain residue numbering system. Novel mutations indicated in bold type, previously reported mutations in plain text. PPK, palmoplantar keratoderma.**Figure 1 Novel mutations in the K6a gene.**

(a) Normal DNA sequence of K6a exon 1, region encoding the helix initiation motif. (b) DNA sequence as shown in (a), derived from patient 1, showing heterozygous mis-sense mutation 520T > G, predicting the amino acid change F174V (or F10V in the 1A domain). (c) Normal DNA sequence of K6a exon 7, region encoding the helix termination motif. (d) DNA sequence as shown in (c), derived from patient 2, showing heterozygous mis-sense mutation 1406T > G, predicting the amino acid change L469R (or L114R in the 2B domain). (e) DNA sequence as shown in (c), derived from patient 3, showing heterozygous mis-sense mutation 1414G > A, predicting the amino acid change E472K (or E117K in the 2B domain). (f) Confirmation of mutation F174V by *HincII* digestion. M, molecular weight markers. Lane 1, digest derived from patient 1 showing digested mutant bands; lanes 2–8, digests derived from normal unrelated controls. (g) Confirmation of mutation L469R by *BpmI* digestion. M, molecular weight markers. Lane 1, digest derived from patient 2 showing undigested mutant band; lanes 2–8, digests derived from normal unrelated controls. (h) Confirmation of mutation E472K by *BseRI* digestion. M, molecular weight markers. Lanes 1, 3–8, digests derived from normal unrelated controls; lane 2, digest derived from patient 3 showing undigested mutant band.

**Mutations in the K6a Helix Initiation Motif****Mutations in the K6a Helix Termination Motif****Restriction digest confirmation of K6a mutations****f K6a F174V Mutation, *HincII* Digests****g K6a L469R Mutation, *BpmI* Digests****h K6a E472K Mutation, *BseRI* Digests**



**Figure 2. Novel mutations in the K16 gene.** (a) Normal DNA sequence of K16 exon 1, region encoding the helix initiation motif. (b) DNA sequence as shown in (a), derived from patient 8, showing heterozygous mis-sense mutation 362T > C, predicting the amino acid change M121T (or M4T in the 1A domain). (c) DNA sequence as shown in (a), derived from patient 9, showing heterozygous mis-sense mutation 383T > A, predicting the amino acid change L128Q (or L11Q in the 1A domain). (d) Confirmation of mutation M121T by *Nla*III digestion. M = molecular weight markers. Lanes 1, 3–8, digests derived from normal unrelated controls; lane 2, digest derived from patient 8 showing undigested mutant band. (e) Confirmation of mutation L128Q by *Stu*I digestion. M = molecular weight markers. Lane 1, digest derived from patient 9 showing digested mutant bands; lanes 2–8, digests derived from normal unrelated controls.



**Figure 3. Novel mutations in the K17 gene.** (a) Normal DNA sequence of K17 exon 1, region encoding the helix initiation motif. (b) DNA sequence as shown in (a), derived from patient 12, showing heterozygous mis-sense mutation 284T > C, predicting the amino acid change L95P (or L11P in the 1A domain). (c) DNA sequence as shown in (a), derived from patient 11, showing overlapping sequence traces due to heterozygous deletion mutation 289delCCT, predicting the amino acid change S97del (or S13del in the 1A domain). (d) DNA sequence as shown in (a), derived from patient 10, showing heterozygous mis-sense mutation 296T > C, predicting the amino acid change L99P (or L15P in the 1A domain). (e) Confirmation of mutations L95P and L99P by *Nci*I digestion. M, molecular weight markers. Lane 1, digest derived from patient 10 showing digested mutant bands; lane 2, digest derived from patient 12 showing digested mutant bands; lanes 3–8, digests derived from normal unrelated controls. (f) Confirmation of mutation S97del by *Sfi*I digestion. M, molecular weight markers. Lane 1, digest derived from patient 11 showing digested mutant bands; lanes 2–8, digests derived from normal unrelated controls.

**Table III.** Three of these were novel mutations: F174V, L469R, and E472K detected in patients 1–3, respectively (**Fig 1a–e; Table III**). A further four patients carried previously reported mutations N171del (patients 4, 6, and 7; Bowden *et al*, 1995) and F174S (patient 5; Smith *et al*, 1999a) in the K6a gene (**Table III**, molecular data not shown). Patients 8 and 9, also diagnosed as PC-1, were found to carry novel heterozygous mis-sense mutations in the K16 gene, M121T and L128Q, respectively (**Fig 2a–c; Table III**). Therefore, all patients diagnosed as having the PC-1 variant were found to carry mutations in the K6a or K16 genes.

A mutation detection strategy for the entire coding region of the functional K17 gene was developed based on long-range PCR. By this means, molecular analysis of the K17 gene was performed in the four patients diagnosed as PC-2 on the basis of pilosebaceous cysts (**Table III**). In one case, the presence of natal teeth was an

additional indicator of PC-2 (patient 12). Three novel K17 mutations were found, L95P, S97del, and L99P in patients 10–12, respectively (**Fig 3a–d; Table III**). The fourth PC-2 case was found to have a recurrent mutation in K17 (R94H), which has previously been reported (Covello *et al*, 1998; Smith *et al*, 1997). Of particular interest was mutation S97del detected in a sporadic case, patient 11. This 3 bp deletion is similar to a 3 bp deletion mutation previously identified in K16 in a sporadic case of PC-1 (Smith *et al*, 1999b).

All novel mutations were excluded from a population of 50 ethnically matched, normal, unrelated individuals by restriction digestion of short PCR products spanning the appropriate regions of the K6a, K16, or K17 genes (**Figs 1f–h, 2d, e, and 3e, f**). Therefore, these defects were excluded as common polymorphisms in the human population by this standard genetic test (Cooper and



Krawczak, 1993). Population screening for the recurrent mutations was performed in the original reports of these defects (Bowden *et al*, 1995; Covello *et al*, 1998; Smith *et al*, 1999a).

## DISCUSSION

**Mutation detection strategies for PC mutation hotspots** Here, we describe eight novel mutations in patients with PC: mutations F174V, E472K, and L469R in K6a; M121T and L128Q in K16; and L95P, S97del, and L99P in K17. In addition, we report five new occurrences of three previously reported mutations, N171del and F174S in K6a and R94H in K17 (data not shown). All the mutations described here occur in the helix boundary motif sequences of these keratins, located at the start of the helix 1A domain and the end of the helix 2B domain. To allow comparison with mutations in other keratins, the 1A and 2B domain residue numbers are shown in **Table III** alongside the codon numbers for these mutations.

Of the recurrent mutations, N171del was identified in one familial and two sporadic cases here (patients 4, 6, and 7) and previous reports of K6a (Bowden *et al*, 1995). The analogous mutation has also been reported in the K4 gene in two cases of white sponge nevus (Rugg *et al*, 1995). This mutation is consistent with DNA polymerase slippage during replication of three tandem CAA repeats present in exon 1 of several type II keratin genes, including K4 and K6a (Cooper and Krawczak, 1993). The novel 3 bp K17 mutation S97del reported here probably occurred through a similar slippage mechanism, as it involves two tandem repeats of CTT (**Fig 3**). Similarly, the other recurrent mutation, R94H in K17, occurs in a CpG-containing arginine codon found in exon 1 of most type I keratins. Deamination of CpG dinucleotides is the most common mechanism underlying point mutations in humans (Cooper and Krawczak, 1993). Thus, genomic instability within the first exon of the K6a and K17 genes probably explains why these are the most common mutations reported in PC.

There are no very obvious causative mechanisms for the other mutations reported here; however, most PC mutations reported to date, including those here, occur in the helix boundary motif sequences of the keratin polypeptides involved. The exceptions are a deletion involving the V1 domain of K16 in a keratinous palmoplantar nevus (Terrinoni *et al*, 2000) and a mutation in the central 2B domain of K16 in a case of late-onset PC (Connors *et al*, 2001). The helix boundary motifs are mutational hotspots for all keratin disorders characterized to date (Fuchs and Cleveland, 1998; Irvine and McLean, 1999) and are encoded by exons 1 and 6 in type I keratin genes and exons 1 and 7 in type II keratin genes. Therefore, in PC like other keratin disorders, mutation analysis should focus first on these exons and only when these are excluded is it worthwhile screening other regions of these genes. It should also be noted that we have previously screened a few cases of PC that do not fall into the PC-1 or PC-2 classifications and detected no mutations in any of the K6a, K6b, K16, or K17 genes (Smith and McLean, unpublished data). Therefore, there are some cases of PC where an unknown gene or genes may be involved.

The keratin genes that are known to be involved in PC are particularly difficult to analyze due to the presence of multiple isogenes and/or pseudogenes (Troyanovsky *et al*, 1992; Takahashi *et al*, 1995; Smith *et al*, 1999b). The mutation detection strategy that we have employed here for K6a and K16 is based on long-range PCR covering all or most of the target gene (Smith *et al*, 1999a, b). For the K17 gene, a specific long-range PCR is reported here, allowing analysis of all exons of this gene in the future. Only one case of a K6b mutation has been reported (Smith *et al*, 1998). Collectively, these methods allow detection of most PC mutations without coamplification of nonfunctional or irrelevant genes.

**Diagnosis and genotype-phenotype correlation in PC** Here, we were able to classify our patients as PC-1 or PC-2 on the basis of the clinical phenotype, as outlined in **Table III**. The most useful distinguishing feature for PC-2 patients is the presence of

pilosebaceous cysts from puberty. Younger patients are more difficult to classify, although very prominent oral lesions are more typical of PC-1 and natal teeth, when present, are indicative of PC-2 (**Table III**). The differences in the PC-1 and PC-2 phenotypes can be largely explained by the difference in expression patterns between the K6a/K16 and K6b/K17 expression pairs (McGowan and Coulombe, 2000). K6b/K17 are expressed at higher levels in the pilosebaceous unit than K6a/K16, explaining the pilosebaceous cysts in PC-2. Conversely, K6a/K16 are more widely expressed in oral epithelia, explaining the greater predominance of oral leukokeratosis in PC-1 (**Table I**).

In conclusion, we report keratin mutations in 13 cases of PC. The genotype-phenotype correlation, mutation clustering data, and long-range PCR methodology presented here should serve as useful guidelines for improved diagnosis and molecular genetic analysis of future cases of this group of keratinizing disorders.

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